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The Biochemistry and Molecular Biology of Chlorophyll Breakdown

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Abstract

Chlorophyll breakdown is one of the most obvious signs of leaf senescence and fruit ripening. The resulting yellowing of leaves can be observed every autumn, and the color change of fruits indicates their ripening state. During these processes, chlorophyll is broken down in a multistep pathway, now termed 'PAO/phyllobilin' pathway, acknowledging the core enzymatic breakdown step catalyzed by pheophorbide *a* oxygenase. It determines the basic linear tetrapyrrole structure of the products of breakdown that are now called 'phyllobilins'. This review provides an update on the PAO/phyllobilin pathway, thereby focusing on recent biochemical and molecular progresses in understanding phyllobilin modifying reactions as the basis for phyllobilin diversity, on evolutionary diversity of the pathway, and on transcriptional regulation of pathway genes.

Introduction

During green organ senescence/maturation as well as during fruit ripening, efficient chlorophyll (Chl) breakdown causes rapid degreening, and consequently unmasks the existence of yellow carotenoids and splendidly highlights the synthesis of diverse anthocyans during the late growing season. Coordinated Chl breakdown is an integral process of senescence/maturation that is developmentally programmed to facilitate the dynamic remobilization of nutrients from senescent organs/tissues to growing organs, reproductive/storage organs in particular (Lim *et al.*, 2007). It has been shown that chloroplasts contain up to 75% of the nitrogen in photosynthetic tissues, with Rubisco accounting for 20-30% of total leaf nitrogen (Makino and Osmond, 1991; Peoples and Dalling, 1988), and up to 95% of seed proteins is synthesized from amino acids derived from the degradation of existing proteins in leaves (Taylor *et al.*, 2010). It is vital, on one hand, to abolish the phototoxicity of free Chl molecules that are uncoupled from light-harvesting Chl-binding complex proteins (LHCs) of the thylakoids in order to maintain cell viability to allow for an efficient remobilization of nutrients in senescent organs/tissues. On the other hand, Chl breakdown has been repeatedly shown to be a prerequisite for the degradation of LHCs in senescent leaves and as such important for accessing this second largest pool of chloroplast nitrogen (Feller *et al.*, 2008; Hörtensteiner and Feller, 2002; Wu *et al.*, 2016). In addition, by-products of Chl degradation are re-used for biosynthetic purposes (Christ and Hörtensteiner, 2014); particularly phytol, which is salvaged into tocopherol during leaf senescence (vom

Dorp *et al*., 2015).

The biochemical pathway of Chl breakdown, the so-called 'PAO/phyllobilin pathway' (Christ and Hörtensteiner, 2014; Kräutler and Hörtensteiner, 2014), has been almost completely revealed based on the characterized enzymes responsible for catalyzing Chl degradation, particularly with the recent demonstration that NONYELLOWING (NYE) proteins, also termed STAY-GREEN (SGR) possess Mg-dechelatase activity (Shimoda *et al*., 2016) (Figure 1). The pathway involves two distinct phases: the early phase deals with the degradation of phototoxic free Chl molecules and their intermediates at the thylakoid membrane within the chloroplast, and the late phase is responsible for modifications of colorless Chl catabolites and their translocation from the chloroplast to the vacuole. Recently, new insight has been obtained on the topology of Chl degradation within the chloroplast as well as on cytosolic modifications of colorless catabolites. Although Chl breakdown can be initiated in various tissues under diverse scenarios, e.g. upon pathogen infection (Mur *et al*., 2010) and under abiotic stresses (Munné-Bosch and Alegre, 2004), massive Chl breakdown occurs mainly in senescent leaves. By taking advantage of the well-characterized *Chl catabolic genes* (*CCGs*), the molecular regulation of Chl breakdown was explored during the past few years, and some of the transcriptional regulatory mechanisms have been revealed in Arabidopsis (*Arabidopsis thaliana*) (Figure 2) as well as in other species (Chen *et al*., 2017; Delmas *et al*., 2013; Gao *et al*., 2016; Li *et al*., 2016; Liang *et al*., 2014; Oda-Yamamizo *et al*., 2016; Qiu *et al*., 2015; Sakuraba *et al*., 2016; Sakuraba *et al*., 2014a; Song *et al*., 2014; Yin *et al*., 2016; Zhang *et al*., 2015; Zhu *et al*., 2015). This review aims to provide an updated summary of progresses in the elucidation of the PAO/phyllobilin pathway and its transcriptional regulation, and to discuss the significance of these progresses as well as remaining lack of knowledge that demands further investigations.

The PAO/phyllobilin pathway of chlorophyll breakdown

Until more than 25 years ago, the fate of Chl during senescence was enigmatic (Hendry *et al.*, 1987). Thus, the identification of compounds that likely derived from Chl and that accumulated in a senescing variety of *Festuca pratensis*, but were absent from a respective stay-green variety, marked an important breakthrough in the field (Matile *et al.*, 1987). In 1991, *Hv*-NCC-1 isolated from senescent barley (*Hordeum vulgare*) leaves was structurally characterized as the first catabolite of Chl that accumulates in the vacuoles of senescing cells

and can be considered as an ultimate product of degradation (Kräutler *et al.*, 1991). This important step fostered the research on Chl breakdown and has led to the identification of currently over 40 breakdown products of Chl from more than 20 plant species that possess the basic common structure of a Chl *a*-derived linear tetrapyrrole, but differ from each other in terms of characteristic side chain modifications and/or stereoisomeric properties. Collectively these catabolites are now termed phyllobilins (Kräutler, 2014).

Chlorophyll is converted to phyllobilins

Phyllobilins can be categorized into two different groups (Kräutler, 2014, 2016). Type I phyllobilins are the historically first identified nonfluorescent Chl catabolites (NCCs) that structurally are 1-formyl, 19-oxobilins (see Figure 1, for atom and pyrrole ring numbering of phyllobilins), while the only recently discovered type II phyllobilins are 1,19-dioxobilins (Müller *et al.*, 2011), now termed dioxobilin-type nonfluorescent Chl catabolites (DNCCs). Interestingly, the later are similar to biliverdin, the first product of heme degradation, in which the 'northern' meso carbon is lost as CO during the heme oxygenase-catalyzed porphyrin ring opening reaction (Unno *et al.*, 2007). In NCCs and DNCCs, all three remaining methine bridges between the pyrrole rings that remain after porphyrin ring opening are fully reduced, leaving behind a linear tetrapyrrole backbone with an unconjugated electron system. The nonfluorescent NCCs and DNCCs are derived from fluorescent precursors, termed FCCs and DFCCs, respectively, that are isomerized to the nonfluorescent end products by acid-catalyzed tautomerization at pyrrole ring C, the isocyclic ring E and the 'southern'-meso position. This isomerization was shown to be driven by an intramolecular protonation step involving the C12 propionic acid side chain (Oberhuber *et al.*, 2003) and to occur in a pH-dependent manner (Christ *et al.*, 2012) inside the acidic vacuolar sap, the final storage place of the phyllobilins (Hinder *et al.*, 1996; Matile *et al.*, 1988). As mentioned above, DNCCs and NCCs possess characteristic side chain modifications at different peripheral positions. So far, the following functionalizations have been described: (i) hydroxylation at $C3^2$ that can be followed by glycosylation and/or malonylation (R^1 in Figure 1), (ii) demethylation at $O8^4$ (R^2), (iii) dihydroxylation of the C18 vinyl group, that can be followed by glucosylation (R^3), and (iv) hydroxymethylation at either C2 or C4 (R^4). It was been assumed that these modifications are introduced in the fluorescent FCC and/or DFCC precursors before their import into the vacuole and subsequent isomerization to NCCs and DNCCs.

Interestingly, in a few plant species, i.e. banana (*Musa acuminata*) and *Spathiphyllum wallisii*, so-called *hypermodified* FCCs (*hm*FCCs) were identified that persist in senescent leaves or fruits and are not isomerized to the respective NCCs (Kräutler, 2014, 2016). This has been rationalized by functionalization of the C12 propionic acid with different moieties (R^5 in Figure 1) such as daucyl, glucosyl, digalactosyl-glyceryl or glucosyl-(dihydroxyphenyl) ethyl, which dramatically slows down FCC-to-NCC isomerization (Oberhuber *et al.*, 2008). Nevertheless, a few NCCs with a conjugated C12 propionic acid side chain have been described (Moser *et al.*, 2012). Among them is a bicycloglycosidic NCC from *Ulmus glabra* in which a glucose moiety intramolecularly connects $C3^2$ with $C12^3$ (Scherl *et al.*, 2016).

Apart from these type I and type II phyllobilins, small quantities of so-called yellow and pink Chl catabolites (YCCs and PiCCs, respectively) have been identified in senescent leaves from several species (Moser *et al.*, 2009b; Moser *et al.*, 2008b; Ulrich *et al.*, 2011). They are oxidation products of NCCs that have been considered to naturally occur as the result of plant endogenous oxidative activities (Vergeiner *et al.*, 2015) and that might contribute to the color of senescent leaves or ripened fruits (Moser *et al.*, 2012; Moser *et al.*, 2008b). Although so far, DNCC-derived dioxobilin-type YCCs or PiCCs have not been described (Kräutler, 2016), such oxidations are likely not restricted to NCCs.

The biochemistry and topology of chlorophyll breakdown

The biochemical mechanism of Chl breakdown was elucidated stepwise during the last 20 years. With the elucidation of the first phyllobilin structure in 1991 (Kräutler *et al.*, 1991) and inhibition studies (Hörtensteiner *et al.*, 1995), it became obvious that a Fe-dependent oxygenase is responsible for porphyrin ring cleavage, thus determining the common backbone structure of all subsequent intermediary and end products of breakdown. This oxygenase was later identified as a Rieske type monooxygenase that oxygenolytically opens the porphyrin ring of pheophorbide *a*, a Mg^{2+}- and phytol-free intermediate of Chl *a* breakdown, and was thus named PHEOPHORBIDE *a* OXYGENASE (PAO) (Pružinská *et al.*, 2003). Because of the importance of this step in the pathway, it is nowadays referred to as the 'PAO/phyllobilin' pathway of Chl breakdown (Kräutler and Hörtensteiner, 2014). Generally, the PAO/phyllobilin pathway can be divided into two parts. Part I was considered to include all reactions that are involved in the conversion of Chl *b* (via Chl *a*) and Chl *a* to *primary* FCC (*p*FCC) (Christ and Hörtensteiner, 2014). All of these reactions occur inside of the chloroplast and are common to all plant species that have been analyzed for Chl breakdown so far. The

158 recent identification of the C3^2-hydroxylase as a chloroplast inner envelope-located Rieske
159 type oxygenase, named TRANSLOCON AT THE INNER CHLOROPLAST ENVELOPE 55
160 (TIC55) (Hauenstein *et al.*, 2016), and the common occurrence of NCCs and DNCCs that are
161 derived from C3^2-hydroxylated *p*FCC (hydroxy-*p*FCC) calls for including this step in
162 reactions of part I of the PAO/phyllobilin pathway. By contrast, part II describes all further
163 side chain-modifying reactions, all of which take place outside the chloroplast, i.e. at the ER
164 or in the cytosol, and include the non-enzymatic isomerization of fluorescent to
165 nonfluorescent phyllobilins.
166
167 Reactions of part I of the PAO/phyllobilin pathway
168 **Conversion of Chl *b* to Chl *a*** – Except one (Müller *et al.*, 2006), all NCCs and DNCCs that
169 are known up to now derive from Chl *a*. The reason for this intriguing fact is the specificity of
170 two of the downstream Chl catabolic enzymes (CCEs), i.e. the Mg-dechelatases, termed SGR
171 or NYE (the latter name being used in the following) and PAO, for Chl *a* and pheophorbide *a*,
172 respectively, while respective '*b*' pigments are not accepted as substrates (Hörtensteiner *et al.*,
173 1995; Shimoda *et al.*, 2016). Thus, prior to its degradation by the PAO/phyllobilin pathway,
174 Chl *b* must be converted to Chl *a* (Shimoda *et al.*, 2012). This is achieved by the reductive
175 half of the so-called 'chlorophyll cycle', which interconverts chlorophyll(ide) *a* and
176 chlorophyll(ide) *b* (Tanaka and Tanaka, 2011). Chl *b* to Chl *a* reduction is a two-step reaction
177 catalyzed by NONYELLOW COLORING 1 (NYC1) and NYC1-LIKE (NOL), two
178 paralogous Chl *b* reductases that depend on NADPH, and HYDROXYMETHYL CHL *a*
179 REDUCTASE (HCAR), which depends on reduced ferredoxin (Fd) as a source of electrons.
180 Genes encoding respective CCEs were recently identified in rice (*Oryza sativa*) and
181 Arabidopsis (Horie *et al.*, 2009; Kusaba *et al.*, 2007; Meguro *et al.*, 2011; Sato *et al.*, 2009).
182
183 **Mg-dechelation and dephytylation** – As mentioned above, pheophorbide *a* is the substrate
184 of PAO, indicating that Mg-dechelation and dephytylation happen before porphyrin ring
185 cleavage. Since its identification more than a century ago (Willstätter and Stoll, 1913),
186 CHLOROPHYLLASE (CLH) that hydrolyzes Chl to chlorophyllide and phytol was assumed
187 to be involved in Chl degradation during leaf senescence. In favor of this are high *in vitro*
188 activities of CLHs (Arkus *et al.*, 2005; Schelbert *et al.*, 2009) and their wide distribution in
189 higher plants and algae (Drazkiewicz, 1994; Shioi and Sasa, 1986). Since 1999, when the first
190 CLHs were cloned independently by two groups (Jakob-Wilk *et al.*, 1999; Tsuchiya *et al.*,

1999), *CLH* genes have been identified from many plant species with surprising results: only in a few cases were the cloned proteins predicted or experimentally shown to be targeted to the chloroplast (Azoulay-Shemer *et al.*, 2011; Azoulay Shemer *et al.*, 2008), while for example the two Arabidopsis CLHs localize outside the plastid, i.e. in the ER and tonoplast (Hu *et al.*, 2015; Schenk *et al.*, 2007). Originally, this was interpreted as Chl dephytylation to (additionally) occur outside the plastid (Takamiya *et al.*, 2000); however, the involvement of CLHs in leaf senescent-related Chl breakdown was repeatedly challenged (Hörtensteiner, 2006; Hu *et al.*, 2015; Liao *et al.*, 2007; Schelbert *et al.*, 2009; Schenk *et al.*, 2007; Zhou *et al.*, 2007). Recently, CLH (together with Chl) was proposed to play a role as a plant endogenous two component defense system. In this system, CLH gets access to plastid-localized Chl upon cell damage by leaf-chewing herbivores, causing the production of chlorophyllide, which was shown to hamper development of *Spodoptora litura* larvae (Hu *et al.*, 2015).

Using an *in silico prediction* approach, Arabidopsis candidates for a phytol hydrolase that is chloroplast-localized and senescence-regulated were proposed (Schelbert *et al.*, 2009). One of them, now named PHEOPHYTINASE (PPH), was shown to specifically hydrolyze pheophytin and respective mutants were unable to degrade Chl and thus exhibited a stay-green phenotype. PPH was independently identified by two other groups in Arabidopsis (Ren *et al.*, 2010) and rice (Morita *et al.*, 2009). Since then PPH has been characterized in many other plant species and consistently shown to be required for Chl breakdown during leaf senescence (Büchert *et al.*, 2011; Cheng and Guan, 2014; Guyer *et al.*, 2014; Zhang *et al.*, 2016). This solidified the concept that Mg-dechelation precedes dephytylation in the PAO/phyllobilin pathway (Kusaba *et al.*, 2013; Tanaka *et al.*, 2011).

Many activities have been considered in the past to be responsible for Mg-dechelation. These include (i) non-enzymatic loss of the Mg^{2+}-ion as the result of changes in plastidial pH during senescence – Mg is known to be readily lost from Chl under acidic conditions (Saga and Tamiaki, 2012), (ii) involvement of potential Mg-chelators, i.e. heat-stable, low molecular weight compounds termed Mg-chelating substances (Costa *et al.*, 2002; Suzuki and Shioi, 2002), and (iii) Mg-dechelatases (Suzuki and Shioi, 2002; Vicentini *et al.*, 1995). However, the molecular nature of such dechelatases remained enigmatic until recently, because of the unawareness that Chl is the likely substrate for dechelation (Schelbert *et al.*, 2009; Shimoda *et al.*, 2016), not the much more polar chlorophyllide or the often used artificial substrate chlorophyllin (Suzuki and Shioi, 2002; Vicentini *et al.*, 1995). Only very recently, NYE was shown to function as Mg-dechelatase *in vitro* as well as *in vivo* (Shimoda *et al.*, 2016). NYE

has been identified independently by several groups (Armstead *et al.*, 2006, 2007; Jiang *et al.*, 2007; Park *et al.*, 2007; Ren *et al.*, 2007), but mutants that are now known to be deficient in NYE exist since decades or even centuries (Barry, 2009; Hörtensteiner, 2009). Indeed, the green cotyledon variety of pea (*Pisum sativum*) used by Gregor Mendel for the dissection of the laws of genetics in 1866 (Mendel, 1866), has a defect in the *NYE1* gene (Aubry *et al.*, 2008; Sato *et al.*, 2007). In the genome of Arabidopsis, three NYEs are encoded, i.e. the highly homologous NYE1 and NYE2 proteins and NYE/SGR-like (SGRL), which is more distantly related. A major difference of these proteins is the presence in NYE1 and NYE2 of a highly conserved cysteine-rich C-terminal domain of unknown function that is absent in SGRL (Hörtensteiner, 2009). It may explain their distinct substrate specificities: while NYE1 is highly specific-for Chl *a*, SGRL prefers phytol-free chlorophyllide *a* as substrate (Shimoda *et al.*, 2016). Different roles of the three Arabidopsis NYEs have been proposed in the past, some of which in retrospect and in light of their role as Mg-dechelatases may be questioned. Firstly, NYE1 was proposed as a mere scaffold protein whose function is to recruit other CCEs to form a highly dynamic protein complex at LHCII, to allow metabolite channeling of potentially phototoxic Chl breakdown intermediates (Sakuraba *et al.*, 2012). As Mg-dechelatase and first enzyme in Chl *a* degradation, NYE1 still could exert this function to coordinate metabolism downstream of Chl *a*, however its demonstrated interaction with upstream enzymes such as NYC1 or NOL is difficult to rationalize. Secondly, NYE2 has been proposed to have a negative role in Chl breakdown (Sakuraba *et al.*, 2014c), but in retrospect is seems not reasonable why an Mg-dechelatase should act negatively. It has to be noted, however, that this proposed negative role of NYE2 was refuted recently (Wu *et al.*, 2016). Thirdly, SGRL, whose expression is high in seedlings but declines during senescence, was proposed to have a role in stress-related Chl degradation rather than for leaf senescence (Sakuraba *et al.*, 2014b). Its high specificity for chlorophyllide *a* (Shimoda *et al.*, 2016) would require SGRL to function together with a CLH-like enzyme rather than with PPH such as the recently identified CHLOROPHYLL DEPHYTYLASE 1 (Lin *et al.*, 2016). Nevertheless, SGRL was shown to physically interact with PPH (Sakuraba *et al.*, 2014b). In summary, more research is required to determine the exact functions of these NYEs for Mg-dechelation of Chl.

Formation of *p*FCC from pheophorbide *a* – The conversion of pheophorbide *a* to *p*FCC was demonstrated to be a Fd-dependent two-step reaction, because besides Fd, two additional protein fractions were shown to be required for *in vitro p*FCC formation (Hörtensteiner *et al.*,

1995; Rodoni *et al.*, 1997), i.e. a membrane fraction of senescent chloroplasts later shown to be PAO (Pružinská *et al.*, 2003), and a stromal fraction, later identified as red Chl catabolite reductase (RCCR) (Wüthrich *et al.*, 2000). The intermediary product of the PAO reaction, red Chl catabolite (RCC), is metabolically channeled *in vitro*, explaining the simultaneous requirement in respective assays of both enzymes. In line with this, PAO and RCCR were shown to physically interact (Pružinská *et al.*, 2007; Sakuraba *et al.*, 2012).

PAO is a Rieske type monooxygenase with two C-terminally located transmembrane domains that anchors it within the thylakoid membrane (Sakuraba *et al.*, 2012). Using $^{18}O_2$ labeling experiments it was demonstrated that only the oxygen incorporated at the C1 formyl position of *p*FCC is derived from molecular oxygen (Hörtensteiner *et al.*, 1998). Intriguingly, similar experiments with the green alga *Auxenochlorella protothecoides* that efficiently degrades Chl under N-limiting, heterotrophic conditions in the dark (Engel *et al.*, 1996), uncovered the principle same type of mechanism (Curty *et al.*, 1995). In contrast to higher plants, *A. protothecoides* does not further convert RCC, but excretes it into the surrounding medium. Accordingly, RCCR is absent from this green alga as based on recent genome and RNAseq analysis (Gao *et al.*, 2014) (Aubry & Hörtensteiner, unpublished).

RCCR specifically reduces the C15/C16 double bound of RCC, thereby introducing a new stereo center at C16. This reduction occurs in a highly stereospecific manner and was shown to depend on the source of RCCR (Hörtensteiner *et al.*, 2000b; Pružinská *et al.*, 2007), dividing these proteins into type I or type II RCCR. For example the Arabidopsis enzyme (a type I RCCR) produces specifically *p*FCC (Mühlecker *et al.*, 1997), the presumed 'S' stereoisomer (Oberhuber *et al.*, 2008), while the enzymes from bell pepper (*Capsicum annuum*) or tomato (*Solanum lycopersicum*) produce epi-*p*FCC (Mühlecker *et al.*, 2000), i.e. the presumed C16 'R' epimer (Oberhuber *et al.*, 2008). Analysis of chimeric RCCRs composed of Arabidopsis and tomato sequence parts identified a single amino acid residue, which when exchanged with the respective tomato residue, converted Arabidopsis RCCR to a type II enzyme that produces epi-*p*FCC (Pružinská *et al.*, 2007). The stereochemistry introduced by RCCR is reflected in the final phyllobilins, i.e. each of the above described NCCs and DNCCs may, depending on the plant species occur in two C16-isomeric forms. RCCRs are members of the Fd-dependent bilin reductase (FDBR) family that includes ELONGATED HYPOCOTYL 2, i.e. phytochromobilin synthase of higher plants and several bilin reductases of cyanobacteria and algae that catalyze phycobilin biosynthesis (Frankenberg and Lagarias, 2003). Although the 3D structures of RCCR and other FDBRs are

highly similar (Sugishima *et al.*, 2009; Sugishima *et al.*, 2010), i.e. pointing to a similar radical mechanism of electron transfer in RCCR as shown for some other FDBRs (Tu *et al.*, 2004), RCCR exhibits very low sequence similarity with these other FDBRs (Frankenberg *et al.*, 2001).

Mutants in PAO and RCCR were originally identified as ACCELERATED CELL DEATH (ACD) 1 and ACD2, respectively (Greenberg and Ausubel, 1993; Greenberg *et al.*, 1994). PAO and RCCR mutants exhibit a light-dependent cell death phenotype, which was shown to be caused by the accumulation of, respectively, pheophorbide *a* and RCC that are highly photodynamic (Mach *et al.*, 2001; Pružinská *et al.*, 2007; Pružinská *et al.*, 2003; Tanaka *et al.*, 2003; Xodo *et al.*, 2012). Interestingly, however, absence of PAO, but not RCCR (Aubry & Hörtensteiner, unpublished), causes additional light-independent cell death, indicating that besides its high phototoxicity (Xodo *et al.*, 2012) pheophorbide *a* could act as a cell death signaling molecule in the dark (Hirashima *et al.*, 2009). However, pheophorbide *a* unlikely functions as a retrograde signal itself, because it was shown to be unable to leave the plastid (Christ *et al.*, 2012), and other components of this proposed signaling pathway have not yet been identified. Once converted to *p*FCC, the potential phototoxicity of chlorophyll is abolished, and although *p*FCC still has a certain capacity to produce singlet oxygen (Jockusch *et al.*, 2014), this is far below the phototoxicity of pheophorbide *a* or RCC, Thus, formation of *p*FCC by PAO and RCCR can be considered the key step of breakdown that accomplishes the abolishment of Chl phototoxicity as the main purpose for its degradation.

***p*FCC-hydroxylation by TIC55** – All so far described reactions of the first part of the PAO/phyllobilin pathway occur in plastids. The recent identification of TIC55 as $C3^2$ hydroxylase (Hauenstein *et al.*, 2016) adds this reaction to part I, because (i) it commonly occurs in all plant species, as concluded from the presence of $C3^2$-hydroxylated phyllobilins in all investigated species (Hauenstein *et al.*, 2016; Kräutler, 2016), and (ii) the reaction takes place in the plastid as shown by the presence in senescent Arabidopsis chloroplasts of both *p*FCC and hydroxy-*p*FCC (Hauenstein *et al.*, 2016). At first glance, this finding was rather surprising, because TIC55 was identified as a potential component of the protein import machinery at the chloroplast inner envelope (Balsera *et al.*, 2010; Calibe *et al.*, 1997); however, this had been questioned in the past (Boij *et al.*, 2009). Evidences for the role of TIC55 as *p*FCC-hydroxylase came from *in vitro* analyses that showed the requirement for reduced Fd for hydroxylating activity and from the analysis of *tic55* mutants which

exclusively accumulate non-hydroxylated phyllobilins, while in wild type more than 50% of all phyllobilins are hydroxylated (Hauenstein *et al.*, 2016).

Reactions of part II of the PAO/phyllobilin pathway

As mentioned above, over 40 structurally different phyllobilins have been isolated from more than 20 plant species. In the past, phyllobilins were named according to their source, by their type (RCC, FCC, DFCC, NCC, DNCC) and their relative or absolute retention time on a standardized HPLC system (Ginsburg and Matile, 1993; Kräutler, 2014, 2016). However, it turns out that, with this system, constitutionally identical phyllobilins receive different names (see Table 1). Thus, a simplifying nomenclature was proposed recently that classifies phyllobilins according to their type and their monoisotopic mass (Christ *et al.*, 2016). Since LC-MS analysis started to become more widely used for phyllobilin analysis (Christ *et al.*, 2016; Müller *et al.*, 2014; Rios *et al.*, 2015), this seems reasonable. Because of distinct and characteristic fragmentation patterns in tandem MS, different classes of phyllobillins such as NCCs vs DNCCs (Christ *et al.*, 2016) and fluorescent vs nonfluorescent phyllobilins (Hauenstein *et al.*, 2016) can be distinguished. However, this classification system ignores isomeric differences between otherwise identical phyllobilins, as for example C16-epimers. Nevertheless, in the past, structurally identical phyllobilins isolated from different species have obtained different names (see Table 1). Thus, it may be desirable in the future to establish a simple but conclusive nomenclature for phyllobilins that concisely describes their structural features but is not redundant.

The basis of the structural variability of phyllobilins are functionalizations at different side chains that occur species-specifically. Besides the TIC55-catalyzed $C3^2$ hydroxylation, the enzymes of only two further modifications have molecularly been identified.

C1 deformylation – Since their first description in 1991 (Kräutler *et al.*, 1991), NCCs were identified from many plant species and for a long time were considered the exclusive degradation products of Chl. Only a few isolated reports hinted to the occurrence of dioxobilin type forms of phyllobilins, originally named urobilinogenoidic Chl catabolites (Djapic and Pavlovic, 2008; Djapic *et al.*, 2009b; Losey and Engel, 2001). They were considered to be oxidation products of NCC precursors that are, thus, likely formed inside the vacuole (Losey and Engel, 2001). Occurrence of DNCCs and NCCs varies among plant species. For example, *Acer platanoides* exclusively accumulates one single DNCC (Müller *et*

al., 2011) while *Cercidiphyllum japonicum* exclusively accumulates NCCs (Curty and Engel, 1996; Oberhuber *et al.*, 2003). By contrast, Arabidopsis for example forms both NCCs and DNCCs simultaneously, whereby the latter account for over 90% of all phyllobilins found (Christ *et al.*, 2013). This hinted to a dedicated, species-specific activity responsible for DNCC formation. Using a combination of inhibition studies and *in silico* analysis, candidates for such an activity were identified in Arabidopsis (Christ *et al.*, 2013). Indeed, DNCC formation was efficiently inhibited by CO, indicating the involvement of cytochrome P450 monooxygenases (CYPs). Based on mutant analysis and *in vitro* activity determination, CYP89A9 was identified as the sole CYP enzyme responsible for DNCC formation in Arabidopsis. As known from many other CYPs (Schuler *et al.*, 2006), CYP89A9 localizes to the ER, and it catalyzes an oxidative deformylation of FCCs to respective DFCCs (Christ *et al.*, 2013). Intriguingly, CYP89A9 seems to be the sole member of the seven-membered CYP89 subfamily in Arabidopsis, because *cyp89a9* mutants are devoid of DNCCs, but accumulate accordingly more NCCs. Since the mutants do not show any obvious phenotype, phyllobilin deformylation does not seem essential. Thus, it will be interesting to analyze in the future how and why during evolution CYP89A9 was specifically recruited for phyllobilin deformylation in Arabidopsis and whether distinct species that accumulate DNCCs evolved this activity independently from each other.

O8^4 demethylation – Similar to phyllobilin deformylation, demethylation of the C8^2-carboxymethyl group occurs in a species-specific manner. In Arabidopsis, it is catalyzed by the cytosol-located METHYLESTERASE 16 (MES16) (Christ *et al.*, 2012), a member of a 20-membered protein family (Yang *et al.*, 2008), indicating its specific recruitment for Chl breakdown. Based on *in vitro* assays, demethylation has originally been considered to occur at the level of pheophorbide *a*, and be followed by spontaneous C13^2-decarboxylation which leads to the formation of pyropheophorbide *a* (Shioi *et al.*, 1996; Suzuki *et al.*, 2006). However, C8^2-decarboxylated phyllobilins have not been identified up to now and comparison of MES16 activity with pheophorbide *a* and *p*FCC demonstrated the specific stability of the remaining carboxylgroup in the latter (Christ *et al.*, 2012). In addition, in an Arabidopsis *pao1* mutant in which MES16 is mis-targeted to the chloroplast, pyropheophorbide *a* accumulates (Christ *et al.*, 2012). This indicates that the natural substrate of MES16 are cytosolic FCCs and DFCCs. Recently, the crystal structure was resolved at high resolution, indicating that MES16 belongs to the α/β-hydrolase protein superfamily (Li

and Pu, 2016). Besides phyllobilins, MES16 was shown to specifically catalyze the demethylation of methyl-indole acetic acid, but not of other phytohormone methylesters (Christ *et al.*, 2012; Li and Pu, 2016); however, the biological relevance of this MES16 activity remains unknown.

Phyllobilin transport and isomerization – Even before the first structure identification of a phyllobilin, it was proposed that Chl catabolites are stored in the vacuole of senescing barley cells (Matile *et al.*, 1988) and this was confirmed in other plant species (Christ *et al.*, 2012; Hinder *et al.*, 1996; Matile, 1997; Pružinská *et al.*, 2007). In addition and as mentioned above, isomerization of fluorescent precursors to respective NCCs and DNCCs is driven by an acidic pH (Oberhuber *et al.*, 2003), i.e. likely happens inside the vacuole. Finally, transport of phyllobilins into isolated barley vacuoles was demonstrated to be ATP-dependent (Hinder *et al.*, 1996). Although the ATP-binding cassette (ABC) transporters ABCC2 and ABCC3 of Arabidopsis were shown to transport NCCs after expression in yeast (*Saccharomyces cerevisiae*) (Lu *et al.*, 1998; Tommasini *et al.*, 1998), the *in vivo* transporter(s) of phyllobilins at the tonoplast have not been identified. Likewise, export of FCCs from the chloroplast was considered to be ATP-dependent (Matile *et al.*, 1992), but respective transporters have not yet been identified.

How conserved is the PAO/phyllobilin pathway in different tissues?

Visible Chl breakdown, i.e. net loss of green color, does not only occur during natural leaf senescence, but, for example, also in response to different biotic and abiotic stresses. This is seen dramatically during the desiccation of resurrection plants that are capable to fully re-hydrate after the re-supply of water (Tuba *et al.*, 1996). In addition to these leaf-related processes, Chl breakdown also occurs in other tissues of a plant, such as during fruit ripening and seed maturation (Delmas *et al.*, 2013; Moser *et al.*, 2009a; Nakajima *et al.*, 2012). Although the reactions of the PAO/phyllobilin pathway have largely been elucidated during leaf senescence, there is a great body of evidence that points to its involvement also in other tissues and during adverse stress conditions. For example, natural green fruit and seed varieties are known from several species, such as tomato *green flesh*, bell pepper *chlorophyll retainer*, soybean (*Glycine max*) *d1d2*, and several green cotyledon varieties of pea. In each of these cases, *NYE* genes were shown to be mutated (Aubry *et al.*, 2008; Barry and Pandey, 2009; Fang *et al.*, 2014; Sato *et al.*, 2007). Furthermore, phyllobilins have been described

from ripening fruits of many species such as pear (*Pyrus communis*), apple (*Malus domestica*) and banana (Moser *et al.*, 2008a; Moser *et al.*, 2012; Müller *et al.*, 2007), and CCE proteins identified in fruit proteomics studies (Barsan *et al.*, 2010; Wang *et al.*, 2013). Likewise, an involvement of CCEs was shown for different stress conditions that induce degreening. For example, PAO induction and phyllobilin formation occurs during dehydration of resurrection plants (Christ *et al.*, 2014), and a role of PAO was shown for the hypersensitive response of Arabidopsis upon *Pseudomonas syringae* infection (Mur *et al.*, 2010).

All these data indicate high conservation of the PAO/phyllobilin pathway for Chl degradation under diverse conditions and in diverse plant tissues. Nevertheless, there are also some inconsistencies regarding Chl breakdown in leaves as compared to fruits and seeds. For example, tomato and Arabidopsis lines that are deficient in PPH exhibit a stay-green leaf phenotype, but fruit or seed degreening are unaffected (Guyer *et al.*, 2014; Zhang *et al.*, 2014). This hints to the involvement of additional, so far unknown, hydrolytic activities that are required for Chl breakdown in seeds and fruits. It has to be noted that in some instances, like during fruit ripening in *Citrus*, CLHs have been proposed to be involved in dephytylation (Azoulay-Shemer *et al.*, 2011; Azoulay Shemer *et al.*, 2008). At present, it can therefore not be excluded that CLHs might play a role in fruits. Similarly, while senescent leaves of the Arabidopsis *nye1-1* mutant that is deficient in NYE1 stay green during senescence, a block of Chl degradation during seed maturation is only evident in a NYE1/NYE2 double mutant (Delmas *et al.*, 2013; Wu *et al.*, 2016; Zhang *et al.*, 2014), pointing to functional redundancy in seeds that is less obvious in leaves. Also the part II CCEs of the PAO/phyllobilin pathway seem to be differentially active in different Arabidopsis tissues, as can be deduced from the relative abundancies of respective phyllobilins that show major differences between seeds and senescent leaves (Menghini and Hörtensteiner, unpublished).

Evolutionary aspects of the PAO/phyllobilin pathway

Apart from a few isolated and rather unclear reports of Chl degradation products in green algae (Doi *et al.*, 1997; Grabski *et al.*, 2016; Miyake *et al.*, 1995) and the well-studied example of *A. protothecoides*, which has been shown to degrade Chl to RCC-like products that are excreted (Engel *et al.*, 1996; Hörtensteiner *et al.*, 2000a; Oshio and Hase, 1969), phyllobilins have exclusively been identified in Angiosperms. This raises the intriguing question whether the PAO/phyllobilin pathway is conserved within all photosynthetic

organisms or whether Chl breakdown through this pathway evolved rather late and is (with maybe few exceptions) indeed restricted to Angiosperms. Phylogenetic analysis of NYEs, PPH, PAO, RCCR and TIC55 demonstrated that these enzymes are highly conserved within land plants, but sequence similarity drops significantly in lower photosynthetic organisms (Hauenstein *et al.*, 2016; Pružinská *et al.*, 2007; Schelbert *et al.*, 2009; Thomas *et al.*, 2009). Thus, it can be argued that all land plants may be capable to degrade Chl via the PAO/phyllobilin pathway and that the PAO/phyllobilin pathway evolved with the appearance of land plants (Hauenstein *et al.*, 2016). However, experimental support for this is scarce. RCCR homologs of the Gymnosperm *Pinus taeda*, the liverwort *Marchantia polymorpha* and the bryophyte *Physcomitrella patens* were shown to exhibit RCCR activity *in vitro* (Pružinská *et al.*, 2007). In line with the fact that *A. protothecoides* excretes RCC-like catabolites, RCCR is absent from green algae, and evolution of RCCR has been proposed a prerequisite for land colonization of terrestrial plants (Hörtensteiner, 2006).

Despite the presence of highly homologous CCE sequences within lower land plants, phyllobilins have so far not been identified in Gymnosperms, ferns or mosses. It was proposed that phyllobilins may not in all cases be the final products of Chl degradation. Accordingly, amounts of degraded Chl do not always correlate to the amounts of accumulated phyllobilins (Christ *et al.*, 2016; Losey and Engel, 2001) and monopyrrolic degradation products were proposed to occur in senescent barley leaves (Suzuki and Shioi, 1999; Suzuki *et al.*, 1999). It remains to be demonstrated to which extent degradation of Chl beyond the level of phyllobilins may occur, particularly in species like the primitive Gymnosperm *Ginkgo biloba* or the lycophyte *Selaginella moellendorffii* that are capable of leaf senescence but seem to be devoid of phyllobilins (Aubry & Hörtensteiner, unpublished).

Experimental evidence for the presence of CCE activities is also limited in green alga. The activity that produces RCC in *A. protothecoides* is biochemically rather similar to that of higher plants. Thus, both enzymes are monooxygenases that specifically incorporate a molecular oxygen-derived oxygen atom at the C1 formyl group (Curty *et al.*, 1995; Hörtensteiner *et al.*, 1998) and both activities are inhibited by Fe-chelators (Hörtensteiner *et al.*, 2000a; Hörtensteiner *et al.*, 1995). However, the most similar PAO-like enzyme encoded in the genome of *A. protothecoides* (Genbank accession number JAT76993) (Gao *et al.*, 2014), exhibits only 31% sequence identity with Arabidopsis PAO, while it is above 70% within land plants. It remains to be shown whether JAT76993 is a genuine PAO. Recently, an NYE

from *Chlamydomonas reinhardtii* was identified that exhibits Mg-dechelatase activity *in vitro* and after expression in Arabidopsis (Matsuda *et al.*, 2016).

The transcriptional regulation of the PAO/phyllobilin pathway

Since massive Chl breakdown is integral to green organ senescence, its regulation has frequently been investigated concomitantly with the general regulation of senescence. Upon consecutive cloning of *CCGs* during the last almost 20 years, it emerged that many *CCGs* are transcriptionally regulated during senescence. However, only during the past few years, the transcriptional regulation of *CCGs* was specifically explored, especially by yeast one-hybrid screening. This led to the recent identification of quite a few transcription regulators that directly target *CCGs* (Figure 2). Interestingly, almost all of these factors mediate the transcriptional regulation of both Chl breakdown and other senescence processes.

Involvement of EIN3, ORE1, and ERFs in directly activating major CCGs during ethylene-triggered Chl breakdown

The biosynthesis and signaling of ethylene (ET) have long been implicated in the process of degreeing during leaf senescence and fruit ripening (Bleecker *et al.*, 1988; Chao *et al.*, 1997; Grbic and Bleecker, 1995; John *et al.*, 1995; Zacarias and Reid, 1990). Lines of solid evidence include: (i) the octuple Arabidopsis mutant plants of *ACSs* (*1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASES*), encoding the key enzyme catalyzing ethylene biosynthesis, is significantly delayed in degreening and senescence (Tsuchisaka *et al.*, 2009), (ii) EIN2 (ETHYLENE INSENSITIVE 2), the key upstream component of ethylene signaling, was found to upregulate *ORE1* (*ORESARA1*, *NAC092*), a key regulatory gene of degreening and leaf senescence, via negatively regulating *miR164* (*microRNA 164*) that directly targets the mRNA of *ORE1* (Kim *et al.*, 2009), and (iii) EIN3, the downstream target of EIN2, was shown to positively regulate both *ORE1* and *NAP* (*NAC-LIKE, ACTIVATED BY AP3/PI*, *NAC029*) directly (Kim *et al.*, 2014) or *ORE1* alone via inhibiting the expression of *miR164* (Li *et al.*, 2013). Although a few more NAC transcription factors were further identified downstream of ORE1 (Kim *et al.*, 2014), it remained unclear until recently how ethylene signaling directly regulates Chl breakdown during leaf senescence. Qiu and colleagues (Qiu *et al.*, 2015) demonstrated that three major *CCGs*, *NYE1*, *NYC1*, and *PAO*, are the direct targets of EIN3 and ORE1, and, importantly, EIN3 and ORE1 promote the transcription of *NYE1* and

NYC1 additively. Moreover, ORE1 promotes ethylene production by activating the expression of *ACS2*, a major ethylene biosynthesis gene. Therefore, EIN3, ORE1, and ACS2 constitute a feed-forward loop involved in the regulation of ethylene-mediated Chl breakdown during leaf senescence in Arabidopsis (Qiu *et al.*, 2015). More recently, CitERF13, an ethylene responsive factor was identified in *Citrus sinensis*, which, like its homologs in Arabidopsis and tomato (AtERF17 and SlERF16, respectively), acts to promote Chl breakdown, likely through activating the expression of *PPH* (Yin *et al.*, 2016). Above all, ethylene-triggered Chl breakdown is part of the degreening process during natural leaf senescence and fruit maturation.

Involvement of ABI3/ABI5/EEL, ABFs, and NAC016/029 in directly activating major CCGs during abscisic acid-triggered Chl breakdown

It has been repeatedly reported that the level of abscisic acid (ABA) is significantly or even dramatically increased during natural or stress-induced leaf senescence (Breeze *et al.*, 2011; Gepstein and Thimann, 1980; He *et al.*, 2005; Liu *et al.*, 2016). Furthermore, alterations in the endogenous ABA level or exogenous application of ABA or an ABA antagonist, significantly change the onset and/or progression of senescence, which is accompanied by changes in the expression profiles of *CCGs* (Raab *et al.*, 2009; Takasaki *et al.*, 2015; Yang *et al.*, 2014; Ye *et al.*, 2017). Notably, NAP, an important NAC transcription factor involved in the regulation of senescence, mediates ABA-triggered leaf senescence via NAP–SAG113 (a PROTEIN PHOSPHATASE 2C, PP2C) and NAP–AAO3 (ABSCISIC ALDEHYDE OXIDASE 3) regulatory modules in Arabidopsis (Yang *et al.*, 2014; Zhang and Gan, 2012). ABA-triggered leaf senescence has also been found to be mediated by RPK1 (RECEPTOR PROTEIN KINASE 1), MAPKKK18, SNAC-As (stress-responsive NAC transcription factors), NAC016, ABIG1 (ABA INSENSITIVE GROWTH 1), and an ABF (ABA-RESPONSIVE ELEMENT-BINDING FACTOR)/RAV1 (RELATED TO ABA-INSENSITIVE 3/VP1) –ORE1 regulatory module in Arabidopsis (Lee *et al.*, 2011; Liu *et al.*, 2016; Matsuoka *et al.*, 2015; Sakuraba *et al.*, 2016; Takasaki *et al.*, 2015; Zhao *et al.*, 2016).

Recently, the direct regulation of Chl breakdown by ABA signaling components has been explored in Arabidopsis as well as in rice. It was found that ABI5 (ABA INSENSITIVE 5) and EEL (ENHANCED EM LEVEL), two group A bZIP transcription factors that act downstream in the ABA signaling pathway, promote Chl breakdown by directly upregulating *NYE1* and *NYC1* (Sakuraba *et al.*, 2014a), and that NAC016, a newly identified component of

553 ABA signaling, also mediates the regulation of Chl breakdown *via* direct activation of *NYE1*
554 (Sakuraba *et al.*, 2016). By screening for transcriptional regulators of *NYE1*, Gao *et al.* (2015)
555 identified ABF2/3/4 as direct activators of *NYE1/2*, *NYC1*, and *PAO* in ABA-triggered as well
556 as natural leaf senescence (Gao *et al.*, 2016). During seed maturation, ABI3, a B3 domain
557 transcription factor, was shown to directly regulate the expression of *NYE1* and *NYE2* to
558 promote Chl breakdown (Delmas *et al.*, 2013). In rice, OsNAP, an orthologue of AtNAP, was
559 also identified as a key regulator of ABA-triggered and age-dependent leaf senescence, which
560 directly targets *NYE1*, *NYC1*, *NYC3* (*PPH*), and *RCCR* as well as other *senescence-associated*
561 *genes* (*SAGs*) (Liang *et al.*, 2014). This progress suggests that ABA-triggered Chl breakdown
562 might be mainly responsible for degreening during abiotic stress-induced leaf senescence or
563 during seed maturation.
564
565 *Involvement of MYC2/3/4 and NAC019/055/072 in directly activating major CCGs during*
566 *jasmonic acid-triggered Chl breakdown*
567 Although quite a few decades ago, methyl jasmonate was shown to be a much stronger
568 promoter of leaf senescence than ABA (Ueda and Kato, 1980), it was not until the beginning
569 of this century that extensive evidence begun to accumulate for the involvement of
570 endogenous jasmonic acid (JA) synthesis and signaling in regulating senescence (Breeze *et al.*,
571 2011; Castillo and Leon, 2008; He *et al.*, 2002; Lee *et al.*, 2015; Schommer *et al.*, 2008; Shan
572 *et al.*, 2011; Yan *et al.*, 2012). Particularly, JA signaling repressors such as Rubisco activase
573 and WRKY57 and antagonistic regulation of *SAG29* that involves the bHLH subgroup IIIe
574 factors MYC2/3/4 and the bHLH subgroup IIId factors bHLH03/13/14/17 were recently
575 identified to mediate JA-triggered leaf senescence (Jiang *et al.*, 2014; Qi *et al.*, 2015; Shan *et*
576 *al.*, 2011). Strikingly, MYC2/3/4 were demonstrated to directly regulate *NYC1*, *NYE1* and
577 *PAO* to promote Chl breakdown during JA-triggered leaf senescence (Zhu *et al*., 2015) and
578 NAC019/055/072, downstream targets of the MYCs in the JA signaling pathway, were also
579 revealed to directly activate a similar set of *CCGs* (*NYE1/2*, *NYC1*) (Zhu *et al*., 2015). In
580 addition, MYC2 and NAC019 interact at the protein level to synergistically up-regulate the
581 expression of *NYE1* (Zhu *et al*., 2015). These findings suggest a complex hierarchical network
582 of JA signaling in coordinating the regulation of efficient Chl breakdown, which may help to
583 explain chlorosis incurred by pathogen infection.
584
585 *Involvement of PIF4/5 and ABI5/EEL in directly activating and SOC1 in directly repressing*

586 *CCGs during dark-induced Chl breakdown*

587 Light deprivation of green tissues (dark treatment) has been frequently exploited to
588 investigate Chl breakdown and/or senescence processes. On the other hand, light has long
589 been known to inhibit senescence of leaves *via* mediation of its perception and presumably
590 signaling, and, specifically, phytochromes were suggested to be responsible for the
591 senescence-inhibitory effect in light. This was rationalized from the observations that red light
592 pulses were able to retard senescence almost as effective as white light, and the red light
593 effect could be reverted by brief irradiation with far red light (Biswal and Biswal, 1984). The
594 signaling details underlying have remained largely unknown for a long time; however,
595 remarkable progress was made recently in elucidating the regulatory network involving phyB
596 (phytochrome B) and PIFs (PHYTOCHROME-INTERACTING FACTORS) (Liebsch and
597 Keech, 2016). It was shown that upon dark treatment *phyB* mutants exhibit an accelerated
598 yellowing phenotype, whereas phyB overexpresser lines stayed green, indicating that the red
599 light signal that inhibits senescence is mainly perceived by phyB (Sakuraba *et al.*, 2014a).
600 Within the red light signaling pathway, PIF4/5 are major repressors downstream of phyB,
601 which destabilizes them at the posttranslational level, while ELF3 (EARLY FLOWERING 3),
602 a regulator of the circadian clock and flowering time, inhibits *PIF4/5* transcription (Leivar *et*
603 *al.*, 2008; Nusinow *et al.*, 2011; Shin *et al.*, 2009). Notably, during dark-induced senescence,
604 ABI5/EEL and EIN3 were revealed as downstream targets of PIF4/5, which, along with
605 PIF4/5 themselves, regulate *ORE1* (Sakuraba *et al.*, 2014a). PIF4/5, as well as ABI5/EEL,
606 EIN3, and ORE1, were independently demonstrated by different groups to directly regulate
607 two key *CCGs*, *NYE1* and *NYC1* (Qiu *et al.*, 2015; Sakuraba *et al.*, 2014a; Song *et al.*, 2014;
608 Zhang *et al.*, 2015). In addition, PIF4 was found to positively regulate the level of reactive
609 oxygen species as well as ethylene biosynthesis and signaling, and to simultaneously repress
610 the expression of *GLK* (*GOLDEN 2-LIKE*) genes, i.e. chloroplast activity maintenance genes
611 (Oh *et al.*, 2012; Song *et al.*, 2014). These independent investigations collectively reveal PIF4
612 and PIF5, probably also PIF3, as the crucial players in the regulatory network of dark-/light
613 deprivation-induced Chl breakdown and senescence, which hierarchically connects with
614 known key regulators (Liebsch and Keech, 2016).

615 Nevertheless, the regulatory network of dark-induced senescence is far more complex than
616 currently understood, as indicated by the fact that loss-of-functions of almost all SAGs results
617 in stay-green phenotypes during dark-induced senescence. Furthermore, SOC1
618 (SUPPRESSOR OF OVEREXPRESSION OF CO 1), the key integrator of flowering

regulatory pathways, was recently identified as a negative regulator of dark-induced Chl breakdown/senescence (Chen *et al.*, 2017). SOC1 directly inhibits the expression of *NYE1* and *PPH* as well as of *SAG113*, which is involved in ABA-mediated leaf senescence (Zhang and Gan, 2012).

Conclusions

The 'biological enigma' of the fate of Chl (Hendry *et al.*, 1987) has been solved during the last three decades. We now know that a dedicated pathway, the PAO/phyllobilin pathway, is responsible for the degradation of Chl during leaf senescence and fruit ripening of plants. We know the end products of degradation, we know most of the genes and enzymes involved in the pathway, and gene regulatory aspects of the pathway are emerging. Nevertheless, several open questions on the PAO/phyllobilin pathway remain, for example, regarding the molecular identity of the activities that cause the formation of YCCs and PiCCs (Ulrich *et al.*, 2011; Vergeiner *et al.*, 2015) or that catalyze the widely-occurring C18-dihydroxylation (Kräutler *et al.*, 1991) (see Table 1). Furthermore, the fact that a single enzyme of a protein family, such as is the case for Arabidopsis MES16 and CYP89A9, has specifically been recruited to act in phyllobilin modification, favors that there might be a biological role(s) for phyllobilins, beyond their mere disposal as waste products inside the vacuole; however, such a role(s) has not been confirmed yet (Kräutler, 2016). Intriguingly, isolated reports have indicated a posttranscriptional regulation of PAO (Chung *et al.*, 2006; Pružinská *et al.*, 2003), but factors involved in such a regulatory mechanism are still missing. Finally, how all the *CCGs* are regulated so as to act in a coordinated manner also awaits to be elucidated.

Chl degradation through the PAO/phyllobilin pathway might not be the only way of degrading Chl. Indeed, in the past, peroxidases were repeatedly considered to be involved (Hynninen *et al.*, 2010; Johnson-Flanagan and Spencer, 1996; Matile, 1980); however, in none of the cases, have respective degradation products been identified. Recently, several reports (Kashiyama *et al.*, 2012; Kashiyama *et al.*, 2013) demonstrated Chl detoxification in heterotrophic protists during herbivory that does not involve porphyrin ring opening, but ring formation between the C17 propionic acid and ring E. These so-called 13^2,17^3-cyclopheophorbide enols are, in contrast to Chl, pheophytin and pheophorbide, nonfluorescent and incapable to produce singlet oxygen upon light excitation (Kashiyama *et al.*, 2012). Bioluminescent dinoflagellates and Euphausiids such as krill (*Euphausia pacifica*) use Chl-derived linear tetrapyrroles as

652 luciferins (Nakamura *et al.*, 1989; Nakamura *et al.*, 1988). Interestingly, in these cases the
653 porphyrin ring is opened in the 'Western' position. The biochemical and molecular basis for
654 the production of cyclopheophorbide enols or Chl-derived luciferins is unknown (Kashiyama
655 and Tamiaki, 2014; Topalov and Kishi, 2001).
656
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662

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Table 1. Nonfluorescent phyllobilins identified to date.

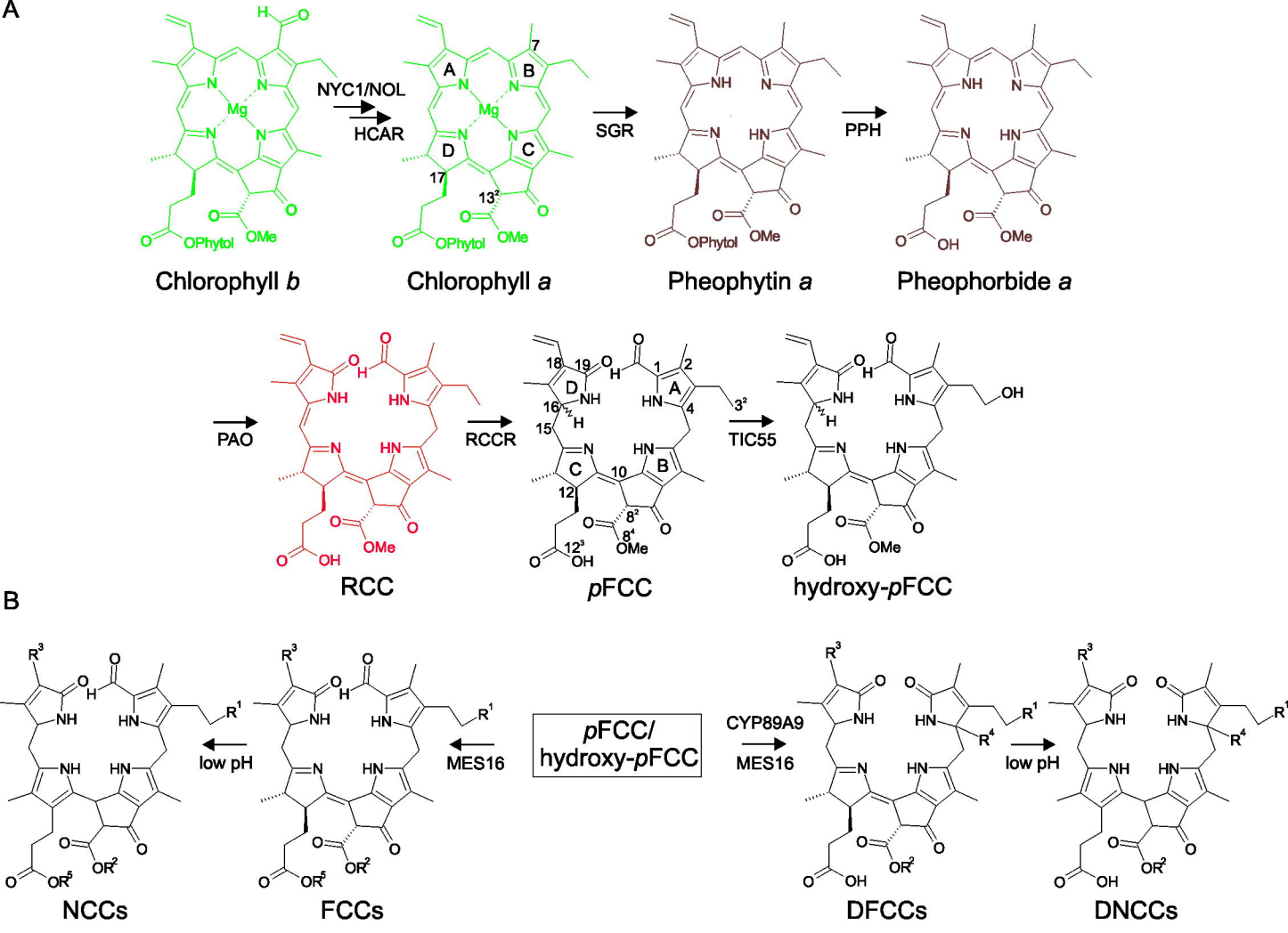
Classifying Name[a]	m/z $[M+H]^+$ calculated	Formula (M)	Side chain modifications[b]					Members of phyllobilin class	Reference[c]
			R^1 ($C3^2$)	R^2 ($O8^4$)	R^3 (C18)	R^4 (C2/C4)	R^5 ($C12^3$)		
DNCC_602	603.2813	$C_{33}H_{38}N_4O_7$	H	H	vinyl	H	H	*At*-DNCC-45[i], *At*-DNCC-48[i]	[1]
DNCC_616	617.2970	$C_{34}H_{40}N_4O_7$	H	CH_3	vinyl	H	H	*At-mes16*-DNCC-47	[2]
DNCC_618	619.2762	$C_{33}H_{38}N_4O_8$	OH	H	vinyl	H	H	*At*-DNCC-1, *At*-DNCC-33; *Bo*-DNCC	[1,3,4]
DNCC_632-1	633.2919	$C_{34}H_{40}N_4O_8$	H	H	vinyl	C4-HM	H	*At*-4HM-DNCC-41	[1]
DNCC_632-2	633.2919	$C_{34}H_{40}N_4O_8$	H	H	vinyl	C2-HM	H	*At*-2HM-*iso*-DNCC-42	[1]
DNCC_632-3	633.2919	$C_{34}H_{40}N_4O_8$	OH	CH_3	vinyl	H	H	*At-mes16*-DNCC-38, *Hvir*-UNCC, *Pa*-DNCC-53, *Pp*-UNCC, *Vv*-UNCC	[2,5-7,31]
DNCC_646-1	647.3075	$C_{35}H_{42}N_4O_8$	H	CH_3	vinyl	C4-HM	H	*At-mes16*-9HM-DNCC-44	[2]
DNCC_646-2	647.3075	$C_{35}H_{42}N_4O_8$	H	CH_3	vinyl	C2-HM	H	*At-mes16*-7HM-*iso*-DNCC-46	[2]
DNCC_650[d]	651.3030	$C_{34}H_{42}N_4O_9$	H	CH_3	DHethyl	H	H	-	[8]
DNCC_666	667.2979	$C_{34}H_{42}N_4O_{10}$	OH	CH_3	DHethyl	H	H	*Ap*-DNCC[j], *Hv*-UCC[k], *Co*-NDCC-2	[9,10,27]
DNCC_780	781.3291	$C_{39}H_{48}N_4O_{13}$	OGlc	H	vinyl	H	H	-	[11]
DNCC_794	795.3447	$C_{40}H_{50}N_4O_{13}$	OGlc	CH_3	vinyl	H	H	-	[11]
DNCC_828[d]	829.3507	$C_{40}H_{52}N_4O_{15}$	OGlc	CH_3	DHethyl	H	H	*Co*-NDCC-1	[27]
DNCC_880[e]	881.3457	$C_{43}H_{52}N_4O_{16}$	OGlcMal	CH_3	vinyl	H	H	-	[8]
NCC_614	615.2813	$C_{34}H_{38}N_4O_7$	H	H	vinyl	H	H	*At*-NCC-5, *Bn*-NCC-4, *Bo*-NCC-2, *Oe*-NCC-4	[4,12, 13,32]
NCC_628	629.2970	$C_{35}H_{40}N_4O_7$	H	CH_3	vinyl	H	H	*Cj*-NCC-2, *Md*-NCC-58, *Oe*-NCC-3, *Pa*-NCC-58, *Pd*-NCC-71, *So*-NCC-5	[14-16, 31,32]
NCC_630[f]	631.2762	$C_{34}H_{38}N_4O_8$	OH	H	vinyl	H	H	*At*-NCC-2, Bn-NCC-3, *Ej*-NCC-3, *Mc*-NCC-49, *Oe*-NCC-1, *So*-NCC-3	[12,16-19,32]
NCC_644	645.2919	$C_{35}H_{40}N_4O_8$	OH	CH_3	vinyl	H	H	*Cj*-NCC-1, *Ej*-NCC-4, *Mc*-NCC-61, *Md*-NCC-2, *Md*-NCC-49 epimer, *Md*-NCC-50, *Oe*-NCC-2, *Pa*-NCC-49, *Pa*-NCC-50, *Pc*-NCC-2, *Pd*-NCC-60, *So*-NCC-4, *Sw*-NCC-58	[14,16,18-22,31,32]
NCC_662[d]	663.3030	$C_{35}H_{42}N_4O_9$	H	CH_3	DHethyl	H	H	-	[8]
NCC_664	665.2823	$C_{34}H_{40}N_4O_{10}$	OH	H	DHethyl	H	H	*Mc*-NCC-26, *So*-NCC-1	[16,18]
NCC_678	679.2979	$C_{35}H_{42}N_4O_{10}$	OH	CH_3	DHethyl	H	H	*Hv*-NCC-1, *Ej*-NCC-1, *Mc*-NCC-42, *Md*-NCC-35, *Pa*-NCC-35, *Pd*-NCC-40, *So*-NCC-2	[14,18,19, 23,24,31]
NCC_716	717.2772	$C_{37}H_{40}N_4O_{11}$	OMal	H	vinyl	H	H	*Bn*-NCC-1	[25]
NCC_730	731.2928	$C_{38}H_{42}N_4O_{11}$	OMal	CH_3	vinyl	H	H	*Ej*-NCC-2	[19]
NCC_788	789.3347	$C_{41}H_{48}N_4O_{12}$	OGlc[h]	CH_3	vinyl	H	H[i]	*Ug*-NCC-53	[26]
NCC_792	793.3291	$C_{40}H_{48}N_4O_{13}$	OGlc	H	vinyl	H	H	*At*-NCC-1, *Bn*-NCC-2, *Bo*-NCC-1	[4,12,17]
NCC_806	807.3447	$C_{41}H_{50}N_4O_{13}$	OGlc	CH_3	vinyl	H	H	*At*-NCC-4, *Mc*-NCC-59, *Md*-NCC-1, *Md*-NCC-47, *Nr*-NCC-2, *Pa*-NCC-47, *Pc*-NCC-1, *Pd*-NCC-56, *Tc*-NCC-2, *Ug*-NCC-43, *Zm*-NCC-2	[12,14,18, 21,26, 28-31]
NCC_826	827.3351	$C_{40}H_{50}N_4O_{15}$	OGlc	H	DHethyl	H	H	*Co*-NCC-2	[27]
NCC_830	831.3089	$C_{42}H_{46}N_4O_{14}$	OH	CH_3	vinyl	H	daucyl	*Mc*-NCC-55[k], *Mc*-NCC-58[k]	[18]
NCC_840	841.3507	$C_{41}H_{52}N_4O_{15}$	OGlc	CH_3	DHethyl	H	H	*Co*-NCC-1, *Md*-NCC-31, *Pa*-NCC-31, *Pa*-NCC-33, *Pd*-NCC-35, *Tc*-NCC-1, *Ug*-NCC-27, *Zm*-NCC-1	[14,26,27, 29-31]
NCC_892	893.3457	$C_{44}H_{52}N_4O_{16}$	OGlcMal	CH_3	vinyl	H	H	*Nr*-NCC-1	[28]
NCC_926[g]	927.3511	$C_{44}H_{54}N_4O_{18}$	OGlcMal	CH_3	DHethyl	H	H	-	[8]
NCC_1002	1003.404	$C_{47}H_{62}N_4O_{20}$	OGlc	CH_3	OH-OGlc	H	H	*Md*-NCC-29, *Pa*-NCC-29, *Pd*-NCC-32	[14,31]

[a]classifying nomenclature for phyllobilins as proposed by Christ at al. (2016). Note that this classification ignores isomeric properties of respective phyllobilin members. [b]positions of modifications as depicted in Figure 1; DHethyl, -CH(OH)-CH_2OH; HM, -CH_2OH; Glc, glucosyl; Mal, malonyl; OH-OGlc, -CH(OH)-CH_2OGlc; [c]references: [1] Süssenbacher *et al*. 2015, [2] Süssenbacher *et al*. 2014, [3] Christ *et al*. 2013, [4] Roiser *et al*. 2015, [5] Djapic & Pavlovic 2008, [6] Djapic *et al*. 2009a, [7] Djapic *et al*. 2009b, [8] Das & Hörtensteiner, unpublished, [9] Losey & Engel 2001, [10] Müller *et al*. 2011, [11] Christ *et al*. 2016, [12] Pruzinska *et al*. 2005, [13] Kräutler & Hörtensteiner 2014, [14] Erhart *et al*. 2016, [15] Oberhuber *et al*. 2003, [16] Berghold *et al*. 2002, [17] Mühlecker *et al*. 1996, [18] Moser *et al*. 2012, [19] Rios *et al*. 2014a, [20] Curty and Engel 1996, [21] Müller *et al*. 2007, [22] Vergeiner *et al*. 2015, [23] Kräutler *et al*. 1991, [24] Oberhuber *et al*. 2001, [25] Mühlecker *et al*. 1993, [26] Scherl *et al*. 2016, [27] Rios *et al*. 2014b, [28] Berghold *et al*. 2004, [29] Berghold *et al*. 2006, [30] Scherl *et al*. 2012, [31] Mittelberger *et al*. 2017, [32] Vergara-Dominguez *et al*. 2016; [d]catabolite identified in barley; [e]catabolite identified in *Lolium perenne*; [f]NCC_630 also for *At*-NCC-3, but there OH is at C2 instead of $C3^2$ (Müller *et al*. 2006); [g]catabolite identified in barley, wheat (*Triticum aestivum*) and *Sorghum bicolor*; [h]a glucose moiety intramolecularly connects $C3^2$ and $C12^3$; [i]potential C4 diastereomers; [j]C10 epimers; [k]two C4 diastereomers of *Hv*-UCC were identified.

Figure legends

Figure 1. The PAO/phyllobilin pathway of chlorophyll breakdown in leaves. **(A)** Reactions of the first part of the pathway from chlorophyll to hydroxy-*p*FCC that take place inside the chloroplast. **(B)** *p*FCC/hydroxy-*p*FCC-modifying reactions of the second part of the pathway that take place outside the chloroplast and lead to the diversity of phyllobilins. For porphyrins and phyllobilins, respectively, pyrrole rings (A-D) and relevant atoms are shown in chlorophyll and *p*FCC, respectively. R^1 to R^5 indicate side chain modifications according to Table 1. See the text for abbreviations of enzyme names. Note, that MES16 and CYP89A9 are specific Arabidopsis enzymes, while all other enzymes are known from different plant species. Also note, that R^4 in DFCCs and DNCCs can alternatively be positioned at C2 (see also Table 1). DFCCs, dioxobilin-type fluorescent chlorophyll catabolites; DNCCs, dioxobilin-type nonfluorescent chlorophyll catabolites; FCCs, formyloxobilin-type fluorescent chlorophyll catabolites; NCCs, formyloxobilin-type nonfluorescent chlorophyll catabolites; *p*FCC, *primary* fluorescent chlorophyll catabolite, RCC, red chlorophyll catabolite.

Figure 2. The up-to-date regulatory network of chlorophyll (Chl) breakdown. **(A)** Regulatory pathways of *Chl catabolic genes* (*CCGs*) by light and respective hormones. Arrows and lines ending in bars indicate promotions and suppressions, respectively; Solid lines represent solid evidence while a dotted line indicates indirect evidence. **(B)** Diagrams showing the known promoters, as well as a repressor in two cases, of individual *CCGs*. See the text for abbreviations and additional details.


A
NYC1/NOL
HCAR
SGR
PPH
Chlorophyll b
Chlorophyll a
Pheophytin a
Pheophorbide a
PAO
RCCR
TIC55
RCC
pFCC
hydroxy-pFCC
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low pH
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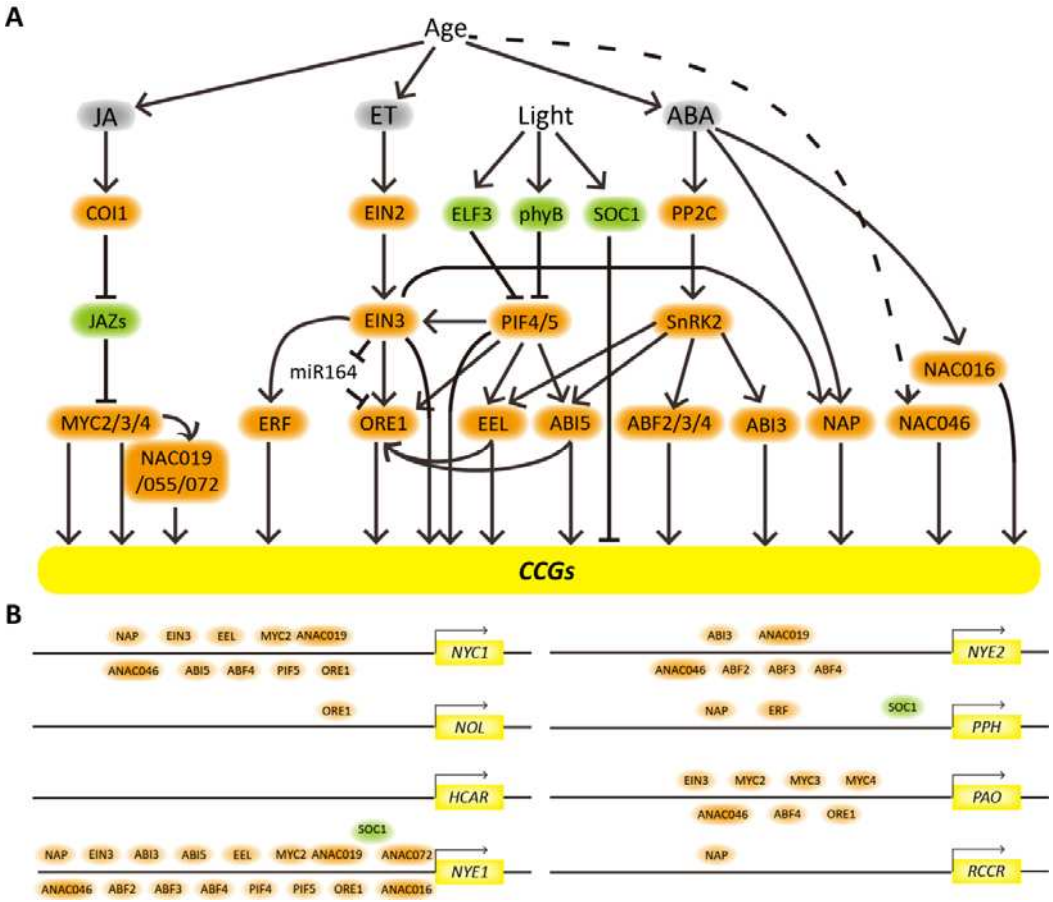
hydroxy-pFCC
CYP89A9
MES16
low pH
NCCs
FCCs
DFCCs
DNCCs


A
Age
JA
ET
Light
ABA
COI1
EIN2
ELF3
phyB
SOC1
PP2C
JAZs
EIN3
PIF4/5
SnRK2
miR164
NAC016
MYC2/3/4
ERF
ORE1
EEL
ABI5
ABF2/3/4
ABI3
NAP
NAC046
NAC019
/055/072
CCGs
B
NAP
EIN3
EEL
MYC2
ANAC019
ANAC046
ABI5
ABF4
PIF5
ORE1
NYC1
ABI3
ANAC019
ANAC046
ABF2
ABF3
ABF4
NYE2
ORE1
NOL
NAP
ERF
SOC1
PPH
HCAR
EIN3
MYC2
MYC3
MYC4
ANAC046
ABF4
ORE1
PAO
SOC1
NAP
EIN3
ABI3
ABI5
EEL
MYC2
ANAC019
ANAC072
ANAC046
ABF2
ABF3
ABF4
PIF4
PIF5
ORE1
ANAC016
NYE1
NAP
RCCR